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Review

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Modulation of auxiliary signals to T cells as a mechanism to induce transplant tolerance

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Summary

Advances in the treatment of transplant rejection, autoimmune disease, allergy, and other conditions of altered immunoregulation have come from our improved knowledge of the multi-faceted nature of lymphocyte activation, incorporating not merely antigen triggering of specific receptors, but a myriad of other accessory signals, all operating within a defined environmental (cytokine) milieu. The review below focuses on just one aspect of this, the ability to manipulate costimulatory signals, or regulatory signals, as a means to induce long-standing immune suppression. Emphasis is placed on the dominant suppression mediated following activation of any one of a number of regulatory signals as a potentially more rational approach to clinical therapy, as the redundancy in costimulatory signals suggests that blockade of any one of these may be unlikely to produce permanent unresponsiveness. The role of regulatory T cells, induced following antigen presentation in the presence of immunoregulatory signals, is also discussed.

Key words: **tolerance • immunoregulation • costimulation • CD200**

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INTRODUCTION

Transplantation is now recognized as the optimal treatment for patients with chronic organ failure, allowing a more normal lifestyle and improved quality of life. Nevertheless, the limits on our basic knowledge of immunobiology leaves immunological graft rejection still as a major drawback to a more widespread application of this therapy. Other major diseases in which understanding natural, not artificial, immunoregulation is key are the autoimmune disorders (rheumatoid arthritis, multiple sclerosis, vasculitis, to name but a few). Current clinical regimens designed to circumvent these problems utilize combinations of a variety of non-specific immunosuppressive drugs. However, in addition to the concomitant toxicity of many of these drugs, these pharmaceuticals also introduce other complications associated with the very non-specificity of the immunosuppression they cause. As a result, these patients have long been recognized as being more susceptible both to opportunistic infection and malignancy.

A potential solution to this problem, which remains the "holy grail" both of transplantation and autoimmunity, is to devise protocols to produce suppression only of (host) responses directed towards the graft/self antigen (so-called **antigen-specific suppression** or tolerance induction). This in turn would decrease and/or eliminate the need for non-specific drug therapy. The development of such technologies has begun to be realized as the body of immunological knowledge has increased to enable an understanding of the nature of self:non-self immunoreactivity at a level of sophistication undreamt of several years ago. The review that follows highlights some of these approaches, focusing in particular on methodologies aimed at altering the response of T cells (and their subsets) to foreign antigen by manipulating not the T cell receptor (TCR):antigen activation signal *per se* (long called signal 1), but one or several of the myriad of accessory signals which are now known to occur which determine the net outcome of antigen triggering.

TOLERANCE AND IMMUNITY: GENERAL THEORY

The key features recognized as the *sine qua non* of the immune system are an anticipatory ability to recognize and respond to the "unexpected" (the adaptability of the immune response) and the ability to make a more rapid and effective immune response the second time the same "foreignness" is encountered (memory). The very plasticity in the development of the receptor repertoire on lymphocytes (achieved by random gene rearrangement of germline elements which encode the lymphocyte receptors) in itself brings with it the problem with which we are concerned herein, the nature of how self:non-self discrimination is achieved since, by necessity, some randomly rearranged receptors will be directed at self structures (potentially leading to "horror autotoxicus").

A number of theoretical explanations which might account for the exquisite regulation of immune reactivity have been considered, although in general they fall under one of three umbrellas: **deletion** of cells bearing self reactive receptors; **antigen-specific suppression** of the developing response by distinct populations of "suppressor cells"; and **anergy**. Development of our understanding of this latter concept has come from a greater appreciation of how lymphocytes become activated by antigen. It is now realized that in addition to a signal delivered by antigen itself, the environmental context in which antigen is experienced is also crucial. As a result, a cell which has the ability to recognize foreignness, but which lacks a nurturing environment, fails to respond appropriately and, like other immune cells, it remembers that failure and cannot be made to respond in future: it has been anergized.

There can be no doubt that there have been enormous gains in our knowledge of the role of each of these mechanisms in tolerance, although not all will be considered in detail in this review. It should be stressed at the outset, however, that it is likely that no mechanism can be used as an all-encompassing explanation, and significant overlap almost certainly occurs under natural conditions. As but one example, coming from our understanding of deletional mechanisms, work on the autoimmune polyglandular syndrome (APS; in humans)[82] and the aire mutation (in animals)[80] has done much to clarify how deletion could be a general mechanism when it was believed (incorrectly as it now appears) that most tissue-specific antigens were not expressed in the thymus (the organ for central deletion of T lymphocytes). A number of groups have established that in fact there is promiscuous expression of a number of such tissue-specific gene products in the thymus (controlled in some manner by products of the aire gene), and failure to express these is indeed associated with a failure of central tolerance (and autoimmunity)[65]. While still not all genes are expressed in the thymus, this markedly promiscuous expression, coupled perhaps with their selective induction of suppressor (or regulatory) cells, by virtue of antigen exposure in the context of a unique antigen-presenting cell (APC) environment (see below), operating to cause bystander suppression to other antigens (see below), might now be invoked as a very general mechanism for tolerance to any self-antigen.

COSTIMULATORY SIGNALS IN T CELL ACTIVATION

$CD8^+$ T cells recognize endogenously synthesized antigen in association with MHC class I[17], while $CD4^+$ T cells usually recognize exogenously acquired antigen in association with MHC class II. However, it was recognized many years ago that professional APCs deliver both an antigenic stimulus to the TCR complex (signal 1) and other costimulatory signals (signal 2). Amongst the known professional APCs are included activated B cells, dendritic cells (DCs), and tissue macrophages[40]. Delivery of signal 1 without signal 2 by non-professional APCs (e.g. resting B cells) was described to induce anergy[15].

Since this early demonstration of the importance of costimulatory signals, a large number of APC--expressed molecules have been described which, along with their respective receptors on T cells, can clearly modulate the outcome of T cell:antigen encounter. It is important to note too that the expression of costimulatory molecules by different APCs is itself not static, but a function of APC maturation and differentiation along with local cytokine production[30, 33, 81]. Many of the costimulatory molecules have been implicated in controlling the early events following antigen encounter, while others may take a more important role in later events (development of memory and/or effector cells). In terms of tolerance induction, the studies discussed below highlight some of the evidence that blockade of costimulation may be one novel approach to immunoregulation.

Role of CD80 (CD86) interactions with CD28 and CTLA4 in tolerance induction

The two APC-expressed ligands, CD80 and CD86, with their receptors (on T cells) CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4), were the first documented costimulatory molecules characterized, and the reader is referred to a review by Salomon and Bluestone[65] for more details. It is known that CTLA4, though expressed in delayed form following T cell activation, has a higher affinity for both CD80 and CD86 than the constitutively expressed CD28. This, coupled with the difference in expression of CD80 and CD86 following APC activation, has generated hypotheses concerning the respective roles for interference with these interactions in T cell activation vs. memory generation, and even led to speculation on a regulatory role for CTLA4. As but one example[72], the magnitude of CD8 T cell memory to LCMV infection in mice was found to be similar in $CD28^{-/-}$ mice compared with $^{+/+}$ mice, and the LCMV-specific memory CD8 T cells in the $^{-/-}$ showed normal homeostatic proliferation *in vivo* while conferring protective immunity. The conclusion reached from these studies was that CD28 signaling is not necessary for the proliferative renewal and maintenance of memory CD8 T cells.

A great deal of effort has gone into examining the effect of blockade of CD80/86 interactions with their counter-receptors on inducing tolerance and thus modulating graft rejection/autoimmunity. Blockade of CD80/CD86 in a primate model was found to prolong graft survival[34, 39], while anti-CD28 treatment also blocked chronic renal rejection in rats[46]. Chitnis et al.[9] reported that blocking B7-1 or CTLA4, but not B7-2, in CD28 knock-out (KO) mice induced experimental autoimmune encephalomyelitis (EAE) disease after a single immunization, suggesting that alternative costimulatory pathways to CD28 could induce EAE. An important role for CD86 in autoimmune disease in the central nervous system (CNS) was confirmed by Zehntner et al.[90], who showed that transgenic expression of CD86 in glial cells was associated with spontaneous development of demyelinating disease.

The majority of the effort at modulating costimulation via CD80/86 has revolved around use of the soluble form of CTLA4, CTLA4Ig. This reagent was established as a molecule able to promote skin allograft survival[52], and indeed, in association with sirolimus, to prolong lung allograft survival in rats[77]. Consistent with this immunoregulatory property of signaling via CTLA4, a monoclonal antibody which itself blocks CTLA4 was reported to enhance the rate of granuloma maturation, $CD4^+$ T cell proliferation, and killing of *Leishmania* in a mouse infectious disease model[92].

Because of the different kinetics of expression of CD80/CD86 and CD28/CTLA4, and the different affinity of CTLA4 and CD28 for the same ligands, a number of workers have examined the effect of early vs. late blockade of these costimulatory interactions on immunity, and have also used the mutant fusion protein Y100F, which selectively binds to CD80 and blocks the CD28-CD80 interaction leaving CD28-CD86 binding intact[51], in an attempt to "tease out" the more important variables in these systems. Late blockade of CD28-B7 T cell costimulation by the fusion protein CTLA4Ig, which binds both CD80 and CD86, attenuated the development of transplant arteriosclerosis, mononuclear cell infiltration, and parenchymal fibrosis in a mouse model[38], and selective blockade of CD80 (using Y100F) was as effective as CTLA4Ig in this regard. In contrast to CTLA4Ig, blockade of CD80 alone by Y100F was ineffective in preventing early graft loss and prolonging graft sur-

vival when given early after transplantation. Somewhat in contrast to these data, in a parent to F1 model of graft-versus-host disease (GVHD) in mice, combined CD80/CD86 blockade beginning at the time of donor cell transfer mimicked results seen with CTLA4Ig and completely abrogated either acute or chronic GVHD by preventing the activation and maturation of donor $CD4^+$ T cells as measured by suppression of acquisition of a memory marker phenotype and cytokine production[44]. While similar results were seen with selective CD86 blockade, the degree of CD4 inhibition was in general less than that seen with combined CD80/CD86 blockade. Perhaps most interesting was the effect of selective CD80 blockade, where chronic GVHD was now converted to acute GVHD, and was associated with the induction of Th1 cytokine production, donor $CD8^+$ T cell activation, and development of anti-host cytotoxic T lymphocytes (CTLs). Because this effect was analogous to that seen with selective CTLA4 blockade, the authors concluded that CD80 was a critical ligand for CTLA4 in mediating the down-regulation of Th1 responses and $CD8^+$ T cell activation, while CD86 was critical for the activation of naive $CD4^+$ T cells to develop either a Th1 or a Th2 cytokine-mediated response.

Role of CD40 interactions with CD154 in tolerance induction

It has been known for some time that CD40:CD154 interactions were also crucial for T cell activation (see[1, 84] for reviews). At least one mechanism was thought to involve CD40L expression on activated T cells signaling APCs via CD40L:CD40 interaction to augment CD80/CD86 expression. This would help explain the synergistic inhibition of graft rejection by the combination of specific monoclonal antibiody (mAb) to CD40 and blockade of CD80/CD86 signaling by CTLA4Ig[45]. It is clear that the mechanism(s) involved are more complicated than this, however. In addition, at least in one reported model, blockade of the CD80/86:CD28 pathway was more effective than blockade of the CD40-CD154 pathway in inhibiting allogeneic pig T cell responses and xenogeneic human anti-pig T cell responses *in vitro*[47].

In an autoimmune disease model (EAE), neuroantigen-specific immune responses were reduced in the absence of CD28, although disease was still induced in the CD28 KO mice, with myelin-specific responses equal to those of wild-type controls and extensive demyelination in the spinal cord, following two immunizations. Treatment of CD28 KO mice with anti-CD154 at the time of immunization significantly reduced DTH responses and prevented this development of EAE[17]. Supporting the preeminence of CD80/86 signaling in tolerance induction, treatment with anti-CD154 at the time of oral administration of ovalbumin did not reverse the induction of tolerance to ovalbumin in either high- or low-dose regimens[10], indicating that tolerance for oral antigen could be established regardless of APC maturation by a CD40--specific mAb. The data from transplant studies using blockade of CD40:CD154 are rather more convincing.

Molano et al.[55] explored the transplantation of C57BL/6 islets into spontaneously nonobese diabetic (NOD) mice, a combination in which both allogeneic and autoimmune components are implicated in graft loss, using treatment with anti-CD154. This antibody was effective in prolonging allograft survival, but did not provide permanent protection from diabetes recurrence. In a study of the autoimmune component of graft loss in spontaneously diabetic NOD mice receiving syngeneic islets from young male NOD mice, a less dramatic delay in diabetes recurrence was observed in antibody-treated recipients compared with controls. A more recent study by Taylor et al.[75] addressed the mechanism by which CD154 might achieve immunosuppression in a GVHD model, and concluded that tolerance induction via costimulatory blockade involved generation of a potent immunoregulatory cell that inhibits both naive and primed alloresponses and was dependent upon cell--to-cell contact to prevent the full activation of the naive or primed cells.

A quite different conclusion was reached by van Maurik et al.[79], using a model of TCR-transgenic $CD4^+$ T cells reactive to the MHC class II alloantigen, $H2A^s$ which allowed direct comparison of the fate of a trace population of $H2A^s$-reactive $CD4^+$ T cells challenged with different forms of alloantigen under conditions of CD40-CD154 costimulation blockade. Intravenous infusion of $H2A^s$ leukocytes in combination with anti-CD154 therapy caused deletion of T cells, while after transplantation of an $H2A^s$ cardiac allograft, T cell responses were unaffected by blocking CD40-CD154 interactions. Combined treatment with donor leukocytes and anti-CD154 therapy was more effective in prolonging the survival of cardiac allografts compared with CD154 mAb treatment alone, consistent, the authors speculate, with the notion that the dominant mechanism by which donor leukocyte infusion and anti-CD154 therapy facilitated allograft acceptance was by deletion of donor-reactive direct-pathway T cells, and NOT by the generation of regulatory cells[79]. In yet another transplant (GVHD) study, blockade of LIGHT, a T cell costimulatory molecule belonging to the tumor necrosis factor (TNF) superfamily, by soluble lymphotoxin β

receptor-Ig (LTβR-Ig: see later discussion) was found to inhibit generation of CTLs to host antigenic disparities and to ameliorate GVHD in a B6 to BDF1 mouse model. The effect was even more pronounced by inclusion of anti-CD154 antibody, though, most interestingly, alloantigen-specific CTLs became anergic and persisted in the tolerized mice as a result of costimulatory blockade (without deletion of induction of regulatory cells)[79].

Role of 4-1BB (CD137) interactions with 4-1BBL in tolerance induction

CD137 is a T cell-expressed costimulatory member of the extended TNFR family whose ligand, 4-1BBL, is expressed on activated APCs. It is known that both human and murine 4-1BBL can stimulate both CD4 and CD8 T cells[85], with increased expansion, cytokine production, and the development of CTLs, though, interestingly, 4-1BBL was most effective when both CD4 and CD8 T cells were included in the cultures. CD28 and 4-IBB synergized in the induction of IL-2 by human T cells, an effect only partially blocked by CTLA4Ig.

Further studies examining the role of CD137 in relation to other costimulatory molecules have established an important role for CD137 in memory-cell generation. Thus CD28 KO mice are impaired in the initial expansion of NP366-374-specific CD8 T cells in response to influenza virus infection, whereas 4--1BB ligand KO mice show no defect in primary T cell expansion to influenza virus, despite a marked decrease later in the primary response[5]. After rechallenge with influenza virus, 4-1BBL KO mice have levels of CD8 T cell expansion which are only the equivalent of a primary response, along with concomitant reduction of CTL effector function. Despite this CTL defect, antibody (Ab) responses, and secondary CD4 T cell responses, are unaffected by 4-1BBL deficiency. Thus, CD28 interactions were deemed crucial for initial T cell expansion, whereas CD137/4-1BBL signaling was essential for the survival and/or responsiveness of the memory CD8 T cell pool, a conclusion reached independently by Dawicki and Watts[12].

A recent study has attempted to further our understanding of the mechanism of CD137:4-1BBL interactions[91], exploring the effect of these interactions on $CD4^+CD25^+$ T regulatory (Treg) cells. It is known that these suppressor cells are themselves anergic, proliferating poorly to mitogenic stimulation in culture, but Zheng et al.[91] described an enhanced proliferation of Treg *in vitro* and *in vivo* following 4-1BB costimulation, in the absence of IL-2 production. The 4-1BB-expanded $CD4^+CD25^+$ T cells are functional, as they remain suppressive to other T cells in coculture.

Other studies have explored the potential mechanisms behind CD137:4-1BBL blockade in immunoregulation. An agonistic monoclonal antibody to CD137 was found to prevent the induction of CTL anergy by soluble antigens, and restored the functions of established anergic CTLs upon reencountering their cognate antigen[86]. Consistent with these findings, the antibody inhibited progressive tumor growth caused by soluble tumor antigen-induced tolerance in a P815R model, and also restored proliferation and effector functions of anergic alloreactive 2C T cells in a bone marrow transplantation model. These authors concluded that ligation of CD137 receptor delivered a regulatory signal for T cell anergy and tolerance[86]. Somewhat paradoxically, Sun et al.[71] reported that the administration of an agonistic anti-CD137 mAb reduced the incidence and severity of EAE[71] and adoptive transfer of T cells from treated mice failed to induce EAE. In contrast, anti-CD137 treatment following adoptive transfer of encephalitogenic T cells did not prevent EAE pathogenesis. Further studies suggested that one explanation for these findings was that anti-CD137 treatment initially promoted the activation and proliferation of antigen (Ag)-specific $CD4^+$ T cells and subsequently increased their probability of undergoing activation-induced cell death[71].

Role of ICOS:ICOS ligand interactions in tolerance induction

Another costimulatory molecule thought to be more relevant at later stages of T cell immunity is the inducible costimulatory (ICOS) molecule, expressed by activated T cells. ICOS has homology to CD28 and CD152 and binds B7h/ B7RP-1, a molecule expressed by APCs with homology to CD80 and CD86. ICOS is rapidly up-regulated on most $CD4^+$ and $CD8^+$ T cells following TCR stimulation, though this increase is reduced in the absence of CD80 and CD86 and can be restored by CD28 stimulation, suggesting that CD28-CD80/CD86 interactions may optimize ICOS expression. When ICOS:B7h interactions were blocked by ICOS-Ig, $CD4^+$ T cells produced more interferon (IFN)-γ and less interleukin (IL)-4 and IL-10 than T cells differentiating in the presence of control Ig, suggesting that ICOS may be particularly important for the development of Th2 cells[53]. Dong et al.[13, 14] generated ICOS KO mice to explore the role of ICOS in T cell function more closely and showed that T cell activation and proliferation were defective in the absence of ICOS, with $ICOS^{-/-}$ T cells failing to produce IL-4 when differentiated *in vitro* or

when primed *in vivo*. This is consistent with a wealth of data suggesting that ICOS is required for humoral immune responses after immunization with several antigens.

Interestingly, ICOS KO mice showed enhanced susceptibility to EAE[13], but a complete resistance to collagen-induced arthritis (CIA), illustrating the complexity of immune regulation by costimulatory molecules[14]. Following immunization with Torpedo acetylcholine receptor (AChR), ICOS KO mice were reported resistant to clinical experimental autoimmune myasthenia gravis development, with less serum AChR-specific Igs and decreased Th1 and Th2 differentiation in response to AChR and the AChR dominant α 46-162 peptide[66]. Further consistent with the role of ICOS in EAE, independent studies showed that ICOS-Ig injection into mice after the first signs of EAE ameliorated clinical disease[69]. More recent studies in a mouse diabetes model have implicated a role for ICOS at the level of development of Treg[31]. Thus BDC2.5 TCR transgenic animals contain a small subset of FoxP3$^+$CD4$^+$CD25$^+$ cells in the pancreas, which express the clonotypic receptor and contain functional regulatory activity as well as higher levels of IL-10 and ICOS than their lymph node counterparts. Blockade of ICOS converted early insulitis to diabetes and disrupted the balance of effector:regulatory T cells, implicating a role for ICOS on maintaining Treg-mediated homeostasis[31].

To investigate the role of ICOS in allograft rejection, Kosuge et al.[41] studied graft survival after BALB/c cardiac transplantation to C3H mice. Anti-ICOS antibody and ICOS-Ig prolonged graft survival time relative to that in untreated mice, while combination therapy with both ICOS-Ig and CTLA4Ig induced long-term acceptance of the cardiac allograft and donor-specific tolerance. Yet another combination therapy protocol was described by Ozkaynak et al.[57]. This group reported that anti-ICOS and an ICOS-Ig fusion protein suppressed intragraft T cell activation and cytokine expression and prolonged allograft survival in a manner similar to that seen in ICOS KO recipients, while in combination with cyclosporin A, anti-ICOS led to permanent engraftment. ICOS--B7RP-I costimulation was required for the development of chronic rejection after CD40:CD154 blockade[57].

Role of OX40 (CD134):OX40 ligand interactions in tolerance induction

An important role for CD134 in costimulation is evident from earlier data by Bansal-Pakala et al.[3] showing that a single dose of an agonistic anti-CD134 (yet another member of the TNF family of receptors) could break an existing state of tolerance in the CD4$^+$ T cell compartment, with subsequent T cell expansion and restoration of normal function. Despite this observation it seems clear that costimulation of CD8$^+$ T cells by CD134 is less potent than that triggered by CD137[74]. Nevertheless, a chimeric fusion protein, OX40L-Fc, which stimulates T cells through OX40, as well as CTLA4Ig were both reported to enhance in equivalent fashion the rate of granuloma maturation, CD4$^+$ T cell proliferation, and killing of *Leishmania*[92]. A similar conclusion was reached in a human experimental model system by Ukyo et al.[78], who studied the effects of anti-OX40L mAb and CTLA4Ig on the proliferative response of CD4$^+$ T cells to allogeneic DCs.

Like many other of the costimualtory molecules described above, CD134 signaling primarily functions to maintain CTL survival during the early rounds of cell division after Ag encounter, rather than being implicated in primary activation signals[2]. Yuan et al.[89] emphasized the same from studies of skin allograft survival in mice, documenting a synergy with CD28:B7 in mediating T cell effector responses during skin allograft rejection. Lee et al.[48] also studied synergistic effects (rather than "stand-alone" effects) of CD134 in CD8 costimulation, noting that enforced dual costimulation through CD137 and CD134, but not through CD40, induced profound specific CD8 T cell clonal expansion, while the response of specific CD4 T cells to dual costimulation was additive rather than synergistic.

Summary of effects of costimulatory blockade

While the list above is not exhaustive, and other receptor:counter-receptor interactions have been implicated in costimulation (generally, these molecules have less avidity than those discussed above), it should nevertheless be apparent that despite the importance of costimulation in T cell triggering, tolerance is not reproducibly induced by blocking costimulation alone. Numerous examples of this have been given already. Typical of these are data in the transplant literature where, while CTLA4Ig has been documented to increase survival of rat and primate cardiac allografts[28, 42] as well as pig xenografts[47], other reports show inhibition of an active process of tolerance induction by simultaneous administration of CTLA4Ig[11, 35]. To some extent these apparently conflicting data can be understood as the complexity of both the redundancy in costimulatory signals (likely of evolutionary benefit in terms of developing immunity to pathogens) and the important role for costim-

ulation in the development of various subpopulations of Treg cells (see earlier). Nevertheless, the inconsistencies seen in various models have resulted in a more recent interest in the possible clinical application of the dominant suppressive signals transduced following interaction with regulatory molecules expressed at the T cell (APC) surface. These are discussed below.

Evidence for the existence of molecules signaling "suppression" at the T cell, APC surface

In a search for novel members of the CD80 costimulatory family, genebank searches uncovered evidence for a number of homologues, which nevertheless seemed not to augment T cell responses, but rather suppress them. The nature of the action of several of the more interesting amongst these is discussed below.

Immunoregulation induced by PD-1:PD-1 ligand interaction

Programmed death (PD)-1 is expressed on anti-CD3--stimulated T cells and anti-IgM plus anti-CD40--stimulated B cells, though undetectable on activated macrophages or DCs. There are two known ligands for PD-1:PD-L1 (B7-H1), which is constitutively expressed on splenic T cells, B cells, macrophages, and DCs and up-regulated on T cells by anti-CD3 stimulation, on macrophages by lipopolysaccharide, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), or IL-4, and on DCs by IFN-γ, GM-CSF, or IL-4; and PD-L2 (B7-DC), whose expression was only inducible on macrophages and DCs on stimulation with IFN-γ, GM-CSF, or IL-4[88].

Tsushima et al.[76] examined the roles of these molecules in mouse hapten-induced contact hypersensitivity, finding that delivery of anti-PD-1 mAb or anti--PD-L1 mAb, but not anti-PD-L2 mAb, at sensitization significantly enhanced and prolonged ear swelling. Anti-PD-1 mAb treatment increased the T cell number of draining lymph nodes, while blockade of PD-L1, but not PD-L2, increased the initial T cell proliferative responses against hapten-pulsed APCs from the draining nodes[76]. In terms of a role for PD--1:PD-L1 interaction in allorecognition, the expression of PD-L1 at the maternal-fetal interface was suggested to reflect its participation in suppression of activated maternal leukocytes[58], while Gao et al.[16] have suggested a role in islet graft rejection.

Studies in an EAE model were reported by Salama et al.[64]. They suggested that the PD-1 pathway plays a key role in regulating peripheral tolerance in murine EAE and was a major contributor to the resistance of disease induction in CD28 KO mice. Thus, following immunization with myelin oligodendrocyte glycoprotein (MOG) a progressive increase in expression of PD-1 and its ligand PD-L1, but not PD-L2, occurred within the CNS of mice with EAE. In both wild-type and CD28 KO mice, PD-1 blockade resulted in accelerated and more severe disease, with increased CNS lymphocyte infiltration and an increased autoimmune response to MOG[64].

A review of receptor and ligand expression patterns, as well as the phenotype of CTLA4 or PD-1 KO mice, supports the hypothesis that these pathways are nonredundant. One hypothesis is that the CD80/86:CTLA4 pathway primarily acts to regulate naive T cell activation in secondary lymphoid organs, while the PD-L/PD-1 pathway acts primarily to regulate T, B, and myeloid cell activation (and/or effector function) at peripheral sites of inflammation[8]. This is reminiscent of our own working hypotheses concerning the role for CD200:CD200R interactions in immune regulation (see below and elsewhere[24, 26]).

Immunoregulation induced by B7H3 (B7RP-2):B7H3 ligand interaction

Both in humans and in mouse systems B7RP-2 (a homologue of the ICOS ligand B7RP-1) is reported to suppress T cell proliferation and cytokine production following activation[49]. Thus, in somewhat analogous fashion to ICOS, regulation (not activation) by B7RP-2 seems to function on activated non--naïve T cells, though the ligand (on activated T cells) for this regulatory molecule remains unidentified. B7RP-2 expression was enhanced by IFN-γ but suppressed by IL-4 in DCs, and B7RP-2 KO mice showed an enhanced susceptibility to EAE, with accumulation of elevated autoantibodies to DNA[70]. In keeping with these observations, anti-B7RP-2 antibody enhanced T cell proliferation and cytokine production *in vitro*, and augmented susceptibility to EAE *in vivo*[59].

Immunoregulation induced by B7x (B7-H4):BTLA ligand interaction

Constitutive cell-surface expression of another putative regulator molecule, B7-H4, is sparse, though the molecule is readily induced on hematopoietic cells[68]. The receptor for B7-H4, BTLA, is upregulated on activated T cells[83]. B7-H4 ligation of T cells causes cell cycle arrest, thus inhibiting growth, cytokine secretion, and development of CTL following T cell activation. In addition, infusion of B7-H4Ig into mice

suppresses antigen-specific T cell responses while blockade of endogenous B7-H4 by anti-B7-H4 augments T cell responses[68]. B7-H4 thus may participate in negative regulation of cell-mediated immunity in peripheral tissues.

It is known that mice with deletion of the gene for the receptor for B7-H4 (BTLA) show an increased susceptibility to EAE[83]. More recently, Han et al.[29] reported on the expression patterns of BTLA and the use of anti-BTLA antibody to modulate immunity. BTLA is expressed by developing B and T cells in the bone marrow and thymus and by all mature lymphocytes, splenic macrophages, and mature, but not immature, bone marrow-derived DCs. While T cells from BTLA KO mice are hyper-responsive to anti-CD3 Ab stimulation, anti-BTLA Ab suppresses T cell activation, consistent with a role for BTLA as a negative regulator of the activation and/or function of various hemopoietic cell types[29].

Immunoregulation induced by members of the GITR:GITR ligand family

There are a number of costimulatory molecules (CD134, CD137) which are members of the extended TNFR family. It was of interest, then, when expression of another member of this family, the glucocorticoid-induced T cell receptor (GITR), was documented on $CD25^+CD4^+$ Treg and proposed to play a key role in the peripheral tolerance mediated by these cells[36], a hypothesis seemingly confirmed by evidence that antibodies to GITR abrogated suppression[54]. However, in sharp contrast to these data, Ronchetti et al.[60] have suggested that the proliferation response of both $CD8^+$ and $CD4^+$ peripheral T cells was potentiated by costimulation via GITR and anti-CD3, suggesting GITR be considered a costimulatory molecule! Furthermore, expression of IL-2Rα and production of IL-2 and IFN-γ were increased more with a GITR costimulus than with anti-CD3 alone. Although Treg proliferation was triggered by the GITR costimulus, this proliferation was paralleled by the loss of the anergic phenotype and suppressor activity. Interestingly, further complicating this issue, Kanamaru et al.[36] reported that GITR costimulation (with anti-CD3) augmented production of IL-10, which resulted in counter-regulation of the enhanced proliferative responses.

Immunoregulation induced by members of the TIM:TIM ligand family

In addition to the role for members (CD134, CD137, GITR) of the extended TNFR family in costimulation or regulation of immune responses, TNF family members (in particular TNF-α, LTα and LTβ) are crucial for normal lymphoid organogenesis[50]. Recently it was reported that amongst the genes associated with LT deficiency was a member of the TIM family, TIM4[67]. Members of the T cell-expressed TIM family (T cell immunoglobulin and mucin domain containing molecule) have also been implicated in regulation of T cell function[43]. The tyrosine-phosphorylation motif in the intracellular domain of TIM3 implied a role in signaling via an unknown ligand, and indeed the interaction of TIM3 with its ligand was reported to be involved in regulating Th1 immunity and tolerance[63], along with autoimmunity[56].

Interestingly, TIM4 is a novel member of this family, apparently not expressed in T cells at all, but in stromal components of the spleen and other lymphoid organs[67]. Nevertheless, the linkage between LT deficiency, Th1/Th2 dysregulation, and TIM4 expression is intriguing. LTα signaling is linked to IgE production, with LT KO mice showing an airway inflammation characteristic of allergic disorders/asthma[37]. These same mice show diminished TIM4 expression, suggesting the latter may be implicated in protection from inflammation in the airways.

Immunoregulation induced by CD200:CD200R interactions

Mapping of the BTLA gene in both mouse and humans confirmed a location between two loci, CD200 and CD200R, already implicated in regulating immune reactivity[18, 24, 29]. While CD200 expression is relatively ubiquitous[4], expression of CD200R is restricted to myeloid cells and T cells[20, 87]. The first evidence for an immunoregulatory role for CD200 came with evidence for a correlation between CD200 over-expression and decreased graft rejection following pre-transplant antigen-specific transfusion via the portal vein[21]. This enhanced survival was in turn abolished by anti-CD200 antibody, and recapitulated by use of an immunoadhesin, CD200Fc[19].

Independent confirmation for immunoregulation by CD200:CD200R interactions came from analysis of CD200 KO mice by Hoek et al.[32]. These mice showed increased susceptibility to autoimmune disease, along with evidence for enhanced endogenous macrophage activation and CD200R expression[32]. More recently, Broderick et al.[6] have shown evidence for a role for CD200 in suppression of autoimmune uveitis, and Truitt's group has suggested a role for CD200-induced regulation in suppression of autoimmune hair loss (alopecia)[61, 62]. These data are consistent with our own observations indicating a role for CD200Fc, or cross-linking anti-CD200R, in suppression of CIA in mice[25].

The mechanism by which CD200:CD200R interactions produce immunoregulation remains elusive. We suggested, based on the tissue expression of the signaling component of this dyad, CD200R, that both an anti-inflammatory effect (at the level of macrophages) and one mediated directly on $CD200R^+$ T cells might be involved[26]. With more extensive characterization of the heterogeneity in the CD200R family, all of which appear to use CD200 as ligand[24], we now speculate that suppression mediated by CD200:CD200R1 interaction might be quite different from that mediated by CD200:CD200R2(R3) interaction. In the latter case, preferential induction of tolerogenic DCs, with subsequent production of Treg, is implicated in decreased graft rejection, while for CD200:CD200R1 a direct suppression of activated T cells bearing CD200R1 is proposed[23]. The importance of CD200:CD200R in maintaining immune homeostasis was implied from additional data showing a role for CD200:CD200R interactions in ensuring successful pregnancy[27], and from work indicating a role in tumor immunization[22], a conclusion supported by studies from Rosenblum et al.[61, 62].

Summary of effects of regulatory molecule signaling

Once again it is important to appreciate that the list of documented regulatory molecules continues to expand, and for some (e.g. CTLA4, GITR) there is some confusion as to whether they should be included within the discussion of costimulation or regulation. However, what seems to be unique to signaling via these regulatory molecules is that despite their redundancy, triggering of any one can suppress the system (dominant tolerance), unlike the "leaky" suppression induced by costimulator blockade (itself a function of the great redundancy in costimulatory signals). This in turn makes some teleological sense in terms of the evolution of the acquired immune system, and also makes manipulation of these signals so much more attractive as candidates for therapeutic intervention. Research over the next few years should highlight whether these theoretical considerations hold true.

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